

This Listing of Claims will replace all prior versions, and listings, of claims in the application:

LISTING OF CLAIMS

Claim 1 (currently amended): A purified, ~~large-scale~~ preparation comprising at least 200 grams of tissue factor pathway inhibitor (TFPI) or TFPI analog molecules, wherein less than ~~12%~~ 2% of the TFPI or TFPI analog molecules are ~~modified species, wherein the modified species include one or more of the following:~~

~~an oxidized TFPI or TFPI analog molecule, as detected by reverse phase chromatography;~~

~~a carbamylated TFPI or TFPI analog molecule, as detected by cation exchange chromatography;~~

~~a deamidated TFPI or TFPI analog molecule, as detected through indirect measurement of isoaspartic acid;~~

~~a TFPI or TFPI analog molecule that comprises a cysteine adduct, as determined by amino acid analysis;~~

~~aggregated TFPI or TFPI analog molecules, as detected by size exclusion chromatography; and~~

~~a misfolded TFPI or TFPI analog molecule, as detected by non denaturing SDS-polyacrylamide gel electrophoresis.~~

Claim 2 (previously presented): The purified preparation of claim 1, wherein less than about 9% of the TFPI or TFPI analog molecules are oxidized.

Claim 3 (currently amended): The purified preparation of claim 1, wherein less than about ~~3%~~  
1% of the TFPI or TFPI analog molecules are carbamylated.

Claim 4 (canceled)

Claim 5 (previously presented): The purified preparation of claim 1, wherein less than about 2%  
of the TFPI or TFPI analog molecules comprise a cysteine adduct.

Claim 6 (previously presented): The purified preparation of claim 1, wherein less than about 3%  
of the TFPI or TFPI analog molecules are aggregated.

Claim 7 (previously presented): The purified preparation of claim 1, wherein less than about 3%  
of the TFPI or TFPI analog molecules are misfolded.

Claim 8 (currently amended): The purified preparation of claim 1, wherein ~~members of the~~  
~~plurality of~~ TFPI molecules have the amino acid sequence shown in SEQ ID NO:1.

Claim 9 (previously presented): The purified preparation of claim 1, wherein the TFPI analog  
molecules are ala-TFPI molecules.

Claim 10 (currently amended): A ~~large-scale~~ pharmaceutical formulation comprising ~~at least~~  
~~200 grams of~~ tissue factor pathway inhibitor (TFPI) or TFPI analog molecules, wherein less than

~~12% 2%~~ of the TFPI or TFPI analog molecules are ~~modified species wherein the modified species include one or more of the following:~~

~~an oxidized TFPI or TFPI analog molecule, as detected by reverse phase chromatography;~~

~~a carbamylated TFPI or TFPI analog molecule, as detected by cation exchange chromatography;~~

~~a deamidated TFPI or TFPI analog molecule, as detected through indirect measurement of isoaspartic acid;~~

~~a TFPI or TFPI analog molecule that comprises a cysteine adduct, as determined by amino acid analysis;~~

~~aggregated TFPI or TFPI analog molecules, as detected by size exclusion chromatography; and~~

~~a misfolded TFPI or TFPI analog molecule, as detected by non denaturing SDS-polyacrylamide gel electrophoresis.~~

Claim 11 (previously presented): The pharmaceutical formulation of claim 10, wherein less than about 3% of the TFPI or TFPI analog molecules are oxidized.

Claim 12 (currently amended): The pharmaceutical formulation of claim 10, wherein less than about ~~3%~~ 1% of the TFPI or TFPI analog molecules are carbamylated.

Claim 13 (canceled)

Claim 14 (previously presented): The pharmaceutical formulation of claim 10, wherein less than about 3% of the TFPI or TFPI analog molecules comprise a cysteine adduct.

Claim 15 (previously presented): The pharmaceutical formulation of claim 10, wherein less than about 3% of the TFPI or TFPI analog molecules are aggregated.

Claim 16 (previously presented): The pharmaceutical formulation of claim 10, wherein less than about 3% of the TFPI or TFPI analog molecules are misfolded.

Claim 17 (currently amended): The pharmaceutical formulation of claim 10, wherein ~~members of the plurality of TFPI or TFPI analog molecules are~~ TFPI molecules ~~that~~ have the amino acid sequence shown in SEQ ID NO:1.

Claim 18 (currently amended): The pharmaceutical formulation of claim 10, wherein ~~members of the plurality of TFPI or~~ TFPI analog molecules are ala-TFPI molecules.

Claim 19 (currently amended): A ~~large-scale~~ pharmaceutical formulation comprising:

~~at least 200 grams of~~ tissue factor pathway inhibitor molecules having an additional amino terminal alanine residue (ala-TFPI), wherein less than ~~42%~~ 2% of the TFPI or TFPI analog molecules are ~~modified species, wherein the modified species include one or more of the following:~~

~~an oxidized ala TFPI molecule, as detected by reverse phase chromatography;~~

a carbamylated ala-TFPI molecule, as detected by cation exchange chromatography;

~~a deamidated ala-TFPI molecule, as detected through indirect measurement of isoaspartic acid;~~

~~an ala-TFPI molecule that comprises a cysteine adduct, as determined by amino acid analysis;~~

~~aggregated TFPI or TFPI analog molecules, as detected by size exclusion chromatography; and~~

~~a misfolded ala TFPI molecule, as detected by non-denaturing SDS-polyacrylamide gel electrophoresis,~~

wherein the pharmaceutical formulation comprises 20 mM sodium citrate, 300 mM L-arginine, and 5 mM methionine, pH 5.5.

Claim 20 (withdrawn-currently amended): A method of producing the purified, ~~large scale~~ preparation of claim 1, comprising the steps of:

(1) expressing TFPI or a TFPI analog in a rifampicin-resistant *E. coli* host cell, wherein the TFPI or the TFPI analog is encoded on a plasmid comprising the following elements:

- (a) a transcription promoter;
- (b) a ribosome binding site adjacent to the reclac transcription promoter;
- (c) a nucleotide coding sequence that encodes the TFPI or the TFPI analog adjacent to the ribosome binding site;
- (d) a transcription terminator adjacent to the nucleotide coding sequence;
- (e) a replicon;

- (f) an antibiotic resistance gene; and
  - (g) a gene encoding an N-terminal methionine-removing enzyme;
- (2) isolating inclusion bodies containing the TFPI or the TFPI analog from the *E. coli* host cell;
- (3) isolating the TFPI or the TFPI analog from the inclusion bodies to obtain isolated TFPI or TFPI analog;
- (4) refolding the isolated TFPI or TFPI analog to form refolded TFPI or TFPI analog;
- (5) purifying the refolded TFPI or TFPI analog by SP-Sepharose fast flow chromatography in the presence of  $Mg^{++}$  to form a first preparation of purified TFPI or TFPI analog;
- (6) concentrating the first preparation of purified TFPI or TFPI analog to form a first concentrated preparation of purified TFPI or TFPI analog;
- (7) purifying the first concentrated preparation of purified TFPI or TFPI analog by Q-Sepharose HP chromatography to form a second preparation of purified TFPI or TFPI analog;
- (8) purifying the second preparation of purified TFPI or TFPI analog by butyl HIC chromatography to form a third preparation of purified TFPI or TFPI analog;
- (9) purifying the third preparation of purified TFPI or TFPI analog by SP-Sepharose HP chromatography to form a fourth preparation of purified TFPI or TFPI analog;
- (10) concentrating the fourth preparation of purified TFPI or TFPI analog to form a second concentrated preparation of purified TFPI or TFPI analog molecules, wherein less than about 12% of the TFPI or TFPI analog molecules are modified TFPI or TFPI analog molecules.

Claim 21 (withdrawn): The method of claim 20 wherein the transcription promoter is a reclang promoter.

Claim 22 (withdrawn): The method of claim 20 wherein the ribosome binding site is the ribosome binding site from gene 10 of bacteriophage T7.

Claim 23 (withdrawn): The method of claim 20 wherein the nucleotide coding sequence encodes ala-TFPI.

Claim 24 (withdrawn): The method of claim 23 wherein the nucleotide coding sequence is SEQ ID NO:44.

Claim 25 (withdrawn): The method of claim 20 wherein the transcription terminator comprises the nucleotide sequence shown in SEQ ID NO:42.

Claim 26 (withdrawn): The method of claim 20 wherein the replicon comprises a pBR322 origin of replication.

Claim 27 (withdrawn): The method of claim 20 wherein the replicon comprises a rop copy number control gene from pBR322.

Claim 28 (withdrawn): The method of claim 20 wherein the antibiotic resistance gene is streptomycin adenyltransferase.

Claim 29 (withdrawn): The method of claim 20 wherein the N-terminal methionine-removing enzyme is *E. coli* methionine aminopeptidase.

Claim 30 (withdrawn): The method of claim 20 wherein the *E. coli* host cell is MON210 (ATCC Accession No. PTA-5564).

Claim 31 (withdrawn-currently amended): A method of purifying tissue factor pathway inhibitor (TFPI) or TFPI analog molecules to provide the purified, ~~large-scale~~ preparation of claim 1, comprising the steps of:

(1) purifying recombinantly produced TFPI or TFPI analog molecules by SP-Sepharose fast flow chromatography to form a first preparation of purified TFPI or TFPI analog;

(2) concentrating the first preparation of purified TFPI or TFPI analog to form a first concentrated preparation of purified TFPI or TFPI analog;

(3) purifying the first concentrated preparation of purified TFPI or TFPI analog by Q-Sepharose HP chromatography to form a second preparation of purified TFPI or TFPI analog;

(4) purifying the second preparation of purified TFPI or TFPI analog by butyl HIC chromatography to form a third preparation of purified TFPI or TFPI analog;

(5) purifying the third preparation of purified TFPI or TFPI analog by SP-Sepharose HP chromatography to form a fourth preparation of purified TFPI or TFPI analog;

(6) concentrating the fourth preparation of purified TFPI or TFPI analog to form a second concentrated preparation of purified TFPI or TFPI analog molecules, ~~wherein less than about 42% of the TFPI or TFPI analog molecules are modified TFPI or TFPI analog molecules.~~



Claim 32 (withdrawn): The method of claim 31 wherein the SP-Sepharose fast flow chromatography is performed in the presence of  $Mg^{++}$ .

Claim 33 (withdrawn): The method of claim 31 wherein the TFPI or TFPI analog molecules are produced in yeast cells.

Claim 34 (withdrawn): The method of claim 31 wherein the TFPI or TFPI analog molecules are produced in mammalian cells.

Claim 35 (withdrawn): The method of claim 34 wherein the mammalian cells are CHO cells.

Claim 36 (withdrawn): The method of claim 34 wherein the mammalian cells are HepG2 cells.

Claim 37 (withdrawn): The method of claim 34 wherein the mammalian cells are Chang liver cells.

Claim 38 (withdrawn): The method of claim 34 wherein the mammalian cells are SK hepatoma cells.

Claim 39 (withdrawn-currently amended): A method of expressing tissue factor pathway inhibitor (TFPI) or TFPI analog to provide the purified, ~~large-scale~~ preparation of claim 1, comprising:

(1) culturing a rifampicin-resistant *E. coli* host cell in a fermentation medium, wherein the *E. coli* host cell comprises a plasmid having the following elements:

- (a) a transcription promoter;
- (b) a ribosome binding site adjacent to the reclac transcription promoter;
- (c) a nucleotide coding sequence that encodes TFPI or TFPI analog adjacent to the ribosome binding site;
- (d) a transcription terminator adjacent to the nucleotide coding sequence;
- (e) a replicon;
- (f) an antibiotic resistance gene; and
- (g) a gene encoding an N-terminal methionine-removing enzyme;

wherein one liter of the fermentation medium comprises 41 g dextrose, 2.5 g  $(\text{NH}_4)_2\text{SO}_4$ , 4.0 g sodium polyphosphate, 7.0 g  $\text{K}_2\text{SO}_4$ , 1.63 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 2.0 g methionine, 2.0 g glycerol, 0.5 mg  $\text{H}_3\text{BO}_4$ , 0.5 g cobalt chloride, 0.13 g  $\text{CuSO}_4 \cdot 6\text{H}_2\text{O}$ , 54.0 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 11.0 g  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ , 0.5 g  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 0.02  $\text{NaSeO}_3$ , 22.0 g  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.01 ml concentrated  $\text{H}_2\text{SO}_4$ , and 0.55 ml UCON antifoam.

Claim 40 (withdrawn): The method of claim 39 wherein the transcription promoter is a reclac promoter.

Claim 41 (withdrawn): The method of claim 39 wherein the ribosome binding site is the ribosome binding site from gene 10 of bacteriophage T7.

Claim 42 (withdrawn): The method of claim 39 wherein the nucleotide coding sequence encodes ala-TFPI.

Claim 43 (withdrawn): The method of claim 42 wherein the nucleotide coding sequence is SEQ ID NO:44.

Claim 44 (withdrawn): The method of claim 39 wherein the transcription terminator comprises the nucleotide sequence shown in SEQ ID NO:42.

Claim 45 (withdrawn): The method of claim 39 wherein the replicon comprises a pBR322 origin of replication.

Claim 46 (withdrawn): The method of claim 39 wherein the replicon comprises a rop copy number control gene from pBR322.

Claim 47 (withdrawn): The method of claim 39 wherein the antibiotic resistance gene is streptomycin adenyltransferase.

Claim 48 (withdrawn): The method of claim 39 wherein the N-terminal methionine-removing enzyme is *E. coli* methionine aminopeptidase.

Claim 49 (withdrawn): The method of claim 39 wherein the *E. coli* host cell is MON210 (ATCC Accession No. PTA-5564).

Claim 50 (currently amended): The purified, ~~large-scale~~ preparation of claim 1 comprising 200 grams to 2.4 kilograms of tissue factor pathway inhibitor (TFPI) or TFPI analog.

Claim 51 (currently amended): The purified, ~~large-scale~~ preparation of claim 50 comprising 200-300 grams of tissue factor pathway inhibitor (TFPI) or TFPI analog.

Claim 52 (currently amended): The purified, ~~large-scale~~ preparation of claim 50 comprising 400-600 grams of tissue factor pathway inhibitor (TFPI) or TFPI analog.

Claim 53 (currently amended): The purified, ~~large-scale~~ preparation of claim 50 comprising 600-900 grams of tissue factor pathway inhibitor (TFPI) or TFPI analog.

Claim 54 (currently amended): The purified, ~~large-scale~~ preparation of claim 50 comprising 800-1200 grams of tissue factor pathway inhibitor (TFPI) or TFPI analog.

Claim 55 (currently amended): The purified, ~~large-scale~~ preparation of claim 1, wherein indirect measurement of isoaspartic acid comprises analyzing a byproduct S-adenosyl-homocysteine (SAH) by RP-HPLC, wherein the byproduct is generated from the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to isoaspartic acid, catalyzed by Protein Isoaspartyl Methyl Transferase (PIMT).

Claim 56 (currently amended): The ~~large-scale~~ pharmaceutical formulation of claim 10, wherein indirect measurement of isoaspartic acid comprises analyzing a byproduct S-adenosyl-homocysteine (SAH) by RP-HPLC, wherein the byproduct is generated from the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to isoaspartic acid, catalyzed by Protein Isoaspartyl Methyl Transferase (PIMT).

Claim 57 (currently amended): The ~~large-scale~~ pharmaceutical formulation of claim 19, wherein indirect measurement of isoaspartic acid comprises analyzing a byproduct S-adenosyl-homocysteine (SAH) by RP-HPLC, wherein the byproduct is generated from the transfer of a methyl group from S-adenosyl-L-methionine (SAM) to isoaspartic acid, catalyzed by Protein Isoaspartyl Methyl Transferase (PIMT).

Claim 58 (new): A method of preparing a pharmaceutical composition comprising tissue factor pathway inhibitor (TFPI) or ala-TFPI molecules, wherein less than 2% of the TFPI or ala-TFPI molecules are carbamylated molecules, as detected by cation exchange chromatography, the method comprising:

- (a) purifying refolded TFPI or ala-TFPI, which has been isolated from inclusion bodies following expression in a host cell, with a sequence of chromatography operations to provide a purified, refolded TFPI or ala-TFPI preparation,
- (b) concentrating and diafiltering the purified, refolded TFPI or ala-TFPI preparation to provide a TFPI or ala-TFPI drug substance, and
- (c) formulating the TFPI or ala-TFPI drug substance into the pharmaceutical composition,

wherein the sequence of chromatography operations comprises two cation exchange chromatography operations, an anion exchange chromatography operation, and a hydrophobic interaction chromatography operation.

Claim 59 (new): The method of claim 58, wherein the sequence of chromatography operations comprises, in order, a first cation exchange chromatography operation, an anion exchange chromatography operation, a hydrophobic interaction chromatography operation, and a second cation exchange chromatography operation.

Claim 60 (new): The method of claim 59, wherein the first cation exchange operation is performed in the presence of urea.

Claim 61 (new): The method of claim 59, wherein the first cation exchange operation is performed using a sodium citrate elution buffer.

Claim 62 (new): The method of claim 58, wherein the TFPI or ala-TFPI drug substance has a protein concentration of about 10 mg/ml.

Claim 63 (new): The method of claim 58, wherein the pharmaceutical composition contains 0.15 mg/ml ala-TFPI, 20 mM sodium citrate, 300 mM L-arginine, and 5 mM methionine and has a pH of 5.5.

Claim 64 (new): The method of claim 58, wherein, in step (b), the purified, refolded TFPI or ala-TFPI preparation is concentrated and diafiltered into a buffer to provide the TFPI or ala-TFPI drug substance.

Claim 65 (new): The method of claim 64, wherein the buffer comprises 300 mM L-arginine and 20 mM sodium citrate and has a pH of 5.5.

Claim 66 (new): The method of claim 64, wherein the TFPI or ala-TFPI drug substance is storage stable at < 60°C for at least 24 months.

Claim 67 (new): The method of claim 58, wherein step (a) comprises:

(a1) purifying the refolded TFPI or ala-TFPI by SP-Sepharose fast flow chromatography to form a first preparation of purified TFPI or ala-TFPI,

(a2) concentrating the first preparation of purified TFPI or ala-TFPI to form a first concentrated preparation of purified TFPI or ala-TFPI,

(a3) purifying the first concentrated preparation of purified TFPI or ala-TFPI by Q-Sepharose HP chromatography to form a second preparation of purified TFPI or ala-TFPI,

(a4) purifying the second preparation of purified TFPI or ala-TFPI by butyl HIC chromatography to form a third preparation of purified TFPI or ala-TFPI, and

(a5) purifying the third preparation of purified TFPI or ala-TFPI by SP-Sepharose HP chromatography to provide the purified, refolded TFPI or ala-TFPI preparation.

Claim 68 (new): The method of claim 67 wherein the SP-Sepharose fast flow chromatography is performed in the presence of  $Mg^{++}$ .

Claim 69 (new): The method of claim 58, wherein step (a) provides the purified, refolded TFPI or ala-TFPI preparation in an amount from 200 grams to 2.4 kilograms.

Claim 70 (new): The method of claim 58, further comprising, prior to step (a),

- (1) expressing the TFPI or ala-TFPI in an *E. coli* host cell,
- (2) isolating inclusion bodies containing the TFPI or ala-TFPI from the *E. coli* host cell,
- (3) isolating the TFPI or ala-TFPI from the inclusion bodies to obtain isolated TFPI or ala-TFPI, and
- (4) refolding the isolated TFPI or ala-TFPI to provide the refolded TFPI or ala-TFPI.

Claim 71 (new): The method of claim 70, wherein the *E. coli* host cell is rifampicin-resistant.

Claim 72 (new): The method of claim 70, wherein the *E. coli* host cell comprises a plasmid comprising:

- (a) a reclk transcription promoter,
- (b) a nucleotide coding sequence that encodes the TFPI or ala-TFPI,
- (c) a transcription terminator adjacent to the nucleotide coding sequence, and
- (d) a plasmid copy number control *rop* gene.



Claim 73 (new): The method of claim 72, wherein the plasmid further comprises an antibiotic resistance gene.

Claim 74 (new): The method of claim 73, wherein the antibiotic resistance gene is an aminoglycoside nucleotidyltransferase gene that confers resistance to streptomycin and spectinomycin.

Claim 75 (new): The method of claim 72, wherein the plasmid further comprises a gene encoding an N-terminal methionine-removing enzyme.